

Multiple liver insults synergize to accelerate experimental hepatocellular carcinoma

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Supporting information 1: High fat diet (HFD) recipe. The HFD was prepared in-house and based on rodent diet no. D12451 (Research Diets New Brunswick, USA).

Ingredients

- 261 g of Casein (Sigma cat no. C7078)
- 224 g of Sucrose (MP Biomedical cat no. 904713)
- 214.8 g of Starch (MP biomedical 102955)
- 51 g of AIN mineral mix (MP biomedical cat no. 905455)
- 57 g of Bran (homebrand Natural Bran)
- 3.4 g of Methionine (Sigma-Aldrich cat no. M9625)
- 23 g of Gelatine (Wards, Altona, Australia)
- 4.6 g of choline bitartrate (Sigma cat no.C1629)
- 14.8 g of AIN vitamins (MP biomedical cat no.960098)
- 6 g of Cholesterol (Sigma Cat no.8503)
- 250 g of room temperature Lard (Yorkfoods natural lard) (Goulburn, Australia)
- 35 ml of safflower oil (Melrose organic Safflower oil) (Mount Waverly, Australia)
- 2 drops of strawberry flavouring (Queen strawberry essence (Alderley, Australia).

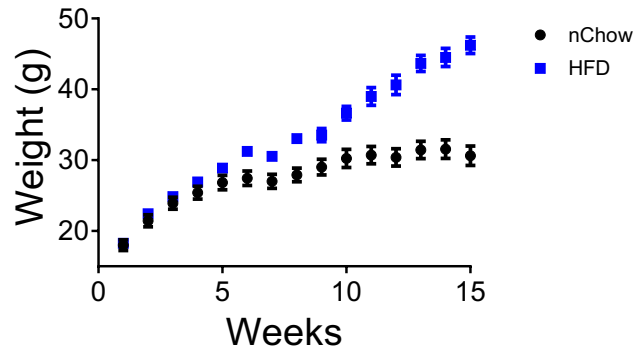
[Regular food items are supermarket – sourced]

Method

Remove Lard from 4 °C and bring to room temperature. In a clean container add Casein, Sucrose, Starch, AIN mineral mix, Bran, Methionine, Gelatine, choline bitartrate, AIN vitamins, Cholesterol and mix through thoroughly. Add room temperature Lard and safflower oil and 2 drops of strawberry flavouring. Mix together until dough - like texture, then store under nitrogen in 4 °C for up to two weeks. Local animal facility sources these items: Mice are housed on Pure-o Cel® bedding (Anderson lab bedding, Ohio, USA) and one wooden chew block (Able Scientific cat no. ASAEC-A) per cage is provided as the chew blocks, extra vitamins and non-edible bedding are essential. The extra vitamins were provided as soluble berry flavoured multivitamins (Berocca, Germany; 1 tablet / L). The drinking water was changed twice per week; once with multivitamins, once without.

Note

Whenever HFD is used, in vivo quality control is done, which is 2 cages of mice running at all times, with weight gain charted weekly (**Supporting information 2**). These are an additional cage of mice treated only with that diet, versus a chow control cage of mice. This is quality control on the diet. Whenever HFD-induced weight gain ceases in these controls the diet and housing conditions are re-evaluated to solve the problem. These mice run for 20 weeks of HFD, then 2 new cages are set up.



Supporting information 2: Diet quality was checked by monitoring weight gain of mice fed *ad libitum* with either the HFD or normal chow (nChow) (n=3-5, Mean and SEM).

Supporting information 3: Antibodies

Antibody	Isotype	Supplier	Catalogue No.	Working dilution
Alpha-SMA	Rabbit	Abcam	Ab32575	1:100
Dipeptidyl peptidase 9	Rabbit	Abcam	Ab42080	1:100
F4/80	Rat	Dr Bertolino	Hybridoma A3-1	neat
Glutathione S- transferase Pi	Rabbit	Santa Cruz	Sc-134469	1:100
Alpha-fetoprotein	Goat	R&D systems	AF5369	1:100
Anti-goat-HRP	Rabbit	Dako	P0449	1:100
Anti-rat-HRP	Goat	Invitrogen	#31470	1:100
Anti-Rabbit-HRP	Goat	Dako	P0448	1:100
CD45 BUV395	Rat	BD Horizon	564279	1:100
CD11b BV421	Rat	BD Horizon	562605	1:100
F4/80 PE-cy7	Rat	Bio Legend	123114	1:100
Ly6C PercP-cy5.5	Rat	Bio Legend	128012	1:100
CD19 PE	Rat	BD Horizon	561736	1:100
Ly6G Alexa647	Rat	Bio Legend	127610	1:100
CD90.2 FITC	Rat	BD Pharmingen	553004	1:100

Supporting information 4: List of gene names and probes.

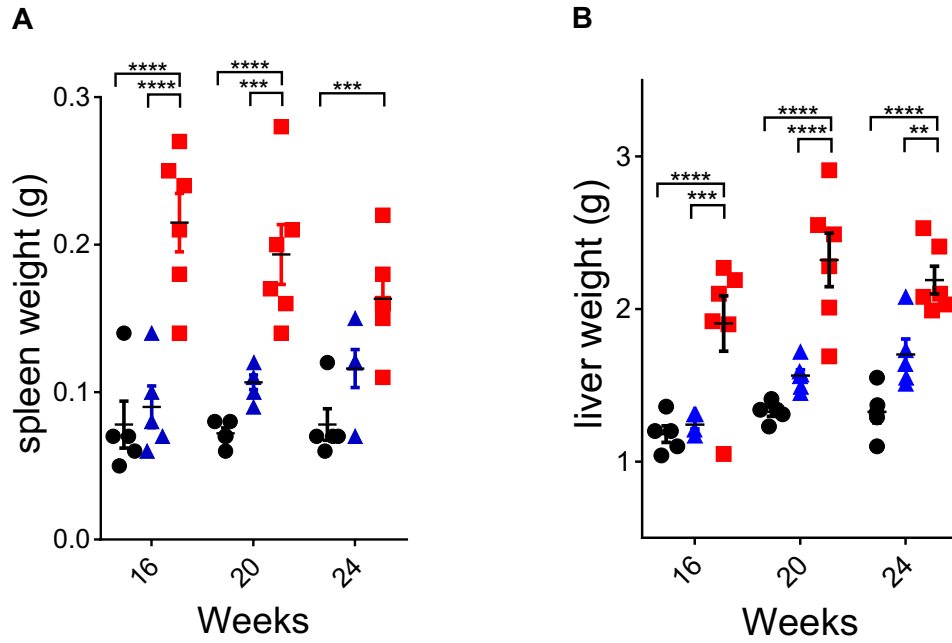
Gene symbol	gene name	gene alias	Primer/probe assay	Gene function	Amplicon length
<i>RN18S</i> (human)	eukaryotic 18S rRNA	18S RNA	Hs99999901_s1	House keeping	187
<i>Afp</i>	alpha fetoprotein		Mm00431715_m1	HCC marker	96
<i>Bax</i>	BCL2-associated X protein		Mm00432051_m1	Apoptosis	84
<i>Bcl2a1a</i>	B cell leukemia/lymphoma 2 related protein A1a	A1, BB218357, Bcl2a1, Bfl-1, Hbpa1	Mm03646861_mH	Apoptosis	112
<i>Bcl2l1</i>	BCL2-like 1	Bcl(X)L, Bcl-XL, Bcl2l, BclX, bcl-x, bcl2-L-1	Mm00437783_m1	Apoptosis	65
<i>Birc2</i>	baculoviral IAP repeat-containing 2	AW146227, Api1, Api2, Birc3, HIAP1, HIAP2, IAP1, IAP2, MIAP1, MIAP2, MIHB, MIHC, RNF48, ciAP1, ciAP2, mciAP1	Mm00431811_m1	NF κ b target. Apoptosis.	90
<i>Birc3</i>	baculoviral IAP repeat-containing 3	AW107670, Api1, Api2, Birc2, HIAP2, IAP1, IAP2, MIAP1, MIAP2, MIHB, MIHC, RNF49, ciAP-1, ciAP-2	Mm01168413_m1	NF κ b target. Apoptosis.	60
<i>Birc5</i>	baculoviral IAP repeat-containing 5	AAC-11, Api4, TIAP, survivin40	Mm00599749_m1	HCC marker	83
<i>Braf</i>	Braf transforming gene	9930012E13Rik, AA120551, AA387315, AA473386, B-raf, Braf-2, Braf2, C230098H17, C87398, D6Ert631e	Mm01165837_m1	Oncogene	94
<i>Casp1</i>	caspase 1	ICE, I11bc	Mm00438023_m1	Apoptosis/pyroptosis	99
<i>Ccl2</i>	chemokine (C-C motif) ligand 2	AI323594, HC11, JE, MCAF, MCP-1, MCP1, SMC-CF, Scya2, Sigje	Mm00441242_m1	Chemokine	74
<i>Ccl5</i>	chemokine (C-C motif) ligand 5	MuRantes, RANTES, SISd, Scya5, TCP228	Mm01302427_m1	Chemokine	103
<i>Ccnd1</i>	cyclin D1	AI327039, CycD1, Cyl-1, PRAD1, bcl-1, cD1	Mm00432359_m1	Cancer	58
<i>Ccne1</i>	cyclin E1	AW538188, CycE1	Mm01266311_m1	Cancer	148
<i>Ccr2</i>	chemokine (C-C motif) receptor 2	Cc-ckr-2, Ccr2a, Ccr2b, Ckr2, Ckr2a, Ckr2b, Cmkbr2, mJe-r	Mm00438270_m1	Chemokine	100
<i>Cd163</i>	CD163 antigen	CD163v2, CD163v3	Mm00474091_m1	Macrophage	83
<i>CD21</i>	complement receptor 2	C3DR, Cr2, CD35, Cr-1, Cr-2, Cr1	Mm00801681_m1	Immune system	77
<i>Cd36</i>	CD36 antigen	FAT, GPIV, Scarb3	Mm01135198_m1	Fatty acid transport	112
<i>Cd47</i>	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	9130415E20Rik, AA407862, AI848868, AW108519, B430305P08Rik, IAP, Itgp	Mm00495011_m1	Macrophages	77
<i>Cd64</i>	Fc receptor, IgG, high affinity I	AI323638, AV092959, Fcgr1, FcgammaRI, IGGHAFC	Mm00438874_m1	Macrophages	58
<i>Cd68</i>	CD68 antigen	Lamp4, Scard1, gp110	Mm03047343_m1	Macrophages	109
<i>Cdk1</i>	cyclin-dependent kinase 1	Cdc2, Cdc2a, p34<CDC2>	Mm00772472_m1	Senescence	76
<i>Cdk4</i>	cyclin-dependent kinase 4	Crk3	Mm00726334_s1	Cancer kinase	54
<i>Cdk9</i>	cyclin-dependent kinase 9 (CDC2-related kinase)	PITALRE	Mm01731275_m1	Cancer kinase	150

Supporting information 4 (continued): List of gene names and probes.

Gene symbol	gene name	gene alias	Primer/probe assay	Gene function	Amplicon length
<i>Cdkn2a</i>	cyclin-dependent kinase inhibitor 2A	ARF-INK4a, Arf, INK4a-ARF, Ink4a/Arf, MTS1, Pctr1, p16, p16(INK4a), p16INK4a, p19ARF	Mm00494449_m1	Senescence	55
<i>Col1a2</i>	collagen, type I, alpha 2	AA960264, AI325291, Col1a-2, Cola-2, Cola2, oim	Mm00483888_m1	Extracellular matrix (ECM)	67
<i>Ctgf</i>	connective tissue growth factor	Ccn2, Fisp12, Hcs24, fisp-12	Mm01192933_g1	Growth factor for ECM	67
<i>Cx3cr1</i>	chemokine (C-X3-C motif) receptor 1		Mm00438354_m1	Chemokine	70
<i>Cxcr3</i>	chemokine (C-X-C motif) receptor 3	Cd183, Cmkar3	Mm00438259_m1	Chemokine/Interleukins	58
<i>Foxp3</i>	forkhead box P3	JM2, scurfin, sf	Mm00475162_m1	Immune system	74
<i>Gpc3</i>	glypican 3	OCI-5	Mm00516722_m1	HCC marker. DPP4 ligand	91
<i>Hif1</i>	hypoxia inducible factor 1, alpha subunit	AA959795, HIF1alpha, MOP1, bHLHe78	Mm00468869_m1	Oxidative stress	75
<i>Hprt1</i>	hypoxanthine guanine phosphoribosyl transferase		Mm00446968_m1	House keeping gene	65
<i>Hras</i>	Harvey rat sarcoma virus oncogene	H-ras, Ha-ras, Harvey-ras, Hras-1, Hras1, Kras2, c-H-ras, c-Ha-ras, c-rasHa, ras	Mm01275932_g1	Oncogene	86
<i>Itgax</i>	integrin alpha X	AI449405, Cd11c, Cr4, N418	Mm00498698_m1	Integrin. ECM binding	96
<i>Keap1</i>	kelch-like ECH-associated protein 1	INRF2, mKIAA0132	Mm00497268_m1	Macrophage	71
<i>Klrk1</i>	killer cell lectin-like receptor subfamily K, member 1	D6H12S2489E, NKG2-D, Nkg2d	Mm00473603_m1	Immune system	124
<i>Kras</i>	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	AI929937, K-ras, Ki-ras, Kras-2, Kras2, p21B, ras	Mm00517492_m1	Proto-oncogene, GTPase	125
<i>Lox12</i>	lysyl oxidase-like 2	1110004B06Rik, 4930526G11Rik, 9430067E15Rik, LOX-L2	Mm00804740_m1	Collagen crosslinking	82
<i>Mif</i>	macrophage migration inhibitory factor	GIF, Glif	Mm01611157_gH	Growth factor	177
<i>Myc</i>	myelocytomatosis oncogene	AU016757, Myc2, Niard, Nird, bHLHe39	Mm00487804_m1	Oncogene	89
<i>Nfkbia</i>	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	AI462015, Nfkb1	Mm00477798_m1	Immune system	70
<i>Nfkbib</i>	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, beta	I(Kappa)B(beta), I-kappa-B-beta, IKB-beta, IKappaBbeta, Ikb, IkbB, NF-kappa-BiB, ikB-B	Mm00456853_m1	Immune system	64
<i>Nkap</i>	NFKB activating protein	2610020O08Rik, AA987160, AI849147, AL024236	Mm00482418_m1	Immune system	87
<i>Nqo1</i>	NAD(P)H dehydrogenase, quinone 1	AV001255, Dia4, Dtd, Nmo-1, Nmo1, Nmor1, Ox-1, Ox1, Qr1	Mm01253561_m1	NAD(P)H dehydrogenase, quinone 1	81
<i>Pai1</i>	serine (or cysteine) peptidase inhibitor, clade E, member 1	PAI-1, PAI1, Planh1, serpine1	Mm00435858_m1	ECM angiogenesis	87
<i>Pbx1</i>	pre B cell leukemia homeobox 1	2310056B04Rik, D230003C07Rik, Pbx-1	Mm04207617_m1	Stellate cell	91
<i>Pdk4</i>	pyruvate dehydrogenase kinase, isoenzyme 4	AV005916	Mm01166879_m1	Cancer kinase	66
<i>Pdl1</i>	CD274 antigen	A530045L16Rik, B7h1, Pcdcd111, Pcdcd1lg1, Pdl1	Mm00452054_m1	Programmed death and apoptosis	77
<i>Pten</i>	phosphatase and tensin homolog	2310035O07Rik, A130070J02Rik, AI463227, B430203M17Rik, MMAC1, TEP1	Mm00477208_m1	A tumor suppressor	73
<i>Rpl37a</i>	ribosomal protein L37a		Mm01546394_s1	House keeping gene	111
<i>Spp1</i>	secreted phosphoprotein 1	Osteopontin, 2AR, Apl-1, BNSP, BSPI, Bsp, ETA-1, Eta, OP, Opn, Opnl, Ric, Spp-1	Mm00436767_m1	Immune system	114
<i>Tgfb1</i>	transforming growth factor, beta 1	TGF-beta1, TGFbeta1, Tgfb, Tgfb-1	Mm01178820_m1	Oncogene	59
<i>Tnf</i>	tumor necrosis factor	DIF, TNF-a, TNF-alpha, TNFSF2, TNFalpha, Tnfa, Tnfsf1a	Mm00443258_m1	Immune system	81
<i>Trp53</i>	transformation related protein 53	Tp53, bbl, bfy, bhy, p44, p53	Mm01731290_g1	Tumor protein p53	119
<i>Xiap</i>	X-linked inhibitor of apoptosis	1110015C02Rik, Aipa, Api3, Birc4, IAP3, ILP-1, MIHA	Mm01311594_mH	NF- κ B target gene. Apoptosis	68

Gene symbol	gene name	gene alias	Primer/probe assay	Gene function	Amplicon length
<i>Arg1</i>	arginase, liver	AI, AI256583, Arg-1, PGIF	Mm00475988_m1	Macrophage	65
<i>Bambi</i>	BMP and activin membrane-bound inhibitor	2610003H06Rik	Mm03024088_g1	TGFb signalling	127
<i>Bcl2</i>	BCL2-like 11 (apoptosis facilitator)	1500006F24Rik, Bim, Bod, bcl2-L-11	Mm00437796_m1	Apoptosis	64
<i>Bhlhe40</i>	basic helix-loop-helix family, member e40	Bhlhb2, C130042M06Rik, CR8, Clast5, Dec1, Sharp2, Stra13, Stra14	Mm00478593_m1	Senescence	58
<i>Casp3</i>	caspase 3	A830040C14Rik, AC-3, Apopain, CASP-3, CC3, CPP-32, CPP32, Caspase-3, Lice, SCA-1, Yama, mldy	Mm01195085_m1	Apoptosis	70
<i>Casp9</i>	caspase 9	AI115399, APAF-3, AW493809, CASP-9, Caspase-9, ICE-LAP6, Mch6	Mm00516563_m1	Apoptosis	68
<i>Ccl21</i>	chemokine (C-C motif) ligand 21A (serine)	6CKBAC2, 6Ckine, ALP, AW987545, CKb9, SCYA21a, SLC, Scya21, Scya21b, Tca4, plt	Mm03646971_gH	Chemokine	91
<i>Cdkn2b</i>	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	AV083695, INK4b, MTS2, p15, p15(INK4b), p15INK4b	Mm00483241_m1	Senescence	112
<i>Cxcl10</i>	chemokine (C-X-C motif) ligand 10	C7, CRG-2, INP10, IP-10, IP10, Ifi10, Scyb10, gIP-10, mob-1	Mm00445235_m1	Chemokine	59
<i>Cxcr2</i>	chemokine (C-X-C motif) receptor 2	CD128, CDw128, Cmkar2, Gpcr16, IL-8Rb, IL-8rb, IL8RA, Il8rb, mL-8RH	Mm00438258_m1	Chemokine	67
<i>Cxcr4</i>	chemokine (C-X-C motif) receptor 4	CD184, Cmkar4, LESTR, PB-CKR, PBSF/SDF-1, Sdf1r, b2b220Clo	Mm01292123_m1	Chemokine	99
<i>Egf</i>	epidermal growth factor	AI790464	Mm01316968_m1	Growth factor	64
<i>Egfr</i>	epidermal growth factor receptor	9030024J15Rik, AI552599, Erbb, Errb1, Errp, Wa5, wa-2, wa2	Mm01187858_m1	Growth factor	101
<i>Foxo1</i>	forkhead box O1	AI876417, Afxh, FKHR, Fkhr1, Foxo1a	Mm00490671_m1	Metabolism	86
<i>G6pc</i>	glucose-6-phosphatase, catalytic	AW107337, G6Pase, G6pt, Glc-6-Pase	Mm00839363_m1	Metabolism	116
<i>Gck</i>	glucokinase	GLK, Gk, Gls006, HK4, HKIV, HXKP, Hlb62, MODY2	Mm00439129_m1	Metabolism	69
<i>Hgf</i>	hepatocyte growth factor	C230052L06Rik, HGF/SF, NK1, NK2, SF, SF/HGF	Mm01135184_m1	Growth factor	85
<i>Hnf4a</i>	hepatic nuclear factor 4, alpha	HNF-4, Hnf4, Hnf4alpha, MODY1, Nr2a1, Tcf14	Mm00433964_m1	Growth factor	114
<i>Igf1</i>	insulin-like growth factor 1	C730016P09Rik, Igf-1, Igf-I	Mm00439560_m1	Growth factor	77
<i>Il1b</i>	interleukin 1 beta	IL-1beta, Il-1b	Mm00434228_m1	Immune system	90
<i>Il2ra</i>	interleukin 2 receptor, alpha chain	CD25, Il2r, Ly-43	Mm01340213_m1	Immune system	83
<i>Il6</i>	interleukin 6	Il-6	Mm00446190_m1	Chemokine. Growth	78
<i>IL8</i>	chemokine (C-X-C motif) ligand 8	Il8, Scyb15, lungkine, weche	Mm00441263_m1	Chemokine	66
<i>mTor</i>	mechanistic target of rapamycin (serine/threonine kinase)	2610315D21Rik, AI327068, FRAP, FRAP2, Frap1, RAFT1, RAPT1, flat	Mm00444968_m1	Metabolism	65
<i>Nk</i>	killer cell lectin-like receptor subfamily B member 1C	AI462337, CD161, Ly-59, Ly55c, Ly59, NK-RP1, NK1.1, NKR-P1.9, NKRP1, NKRP140, Nk-1, Nk-1.2, Nk1, Nk1.2, Nkrp1c, ly-55c, Klrb1c	Mm00824341_m1	Immune system	145
<i>NKT</i>	CD1d2 antigen	Cd1d2, CD1.2, Cd1b, Ly-38	Mm00776138_gH	Immune system	120
<i>Nos2</i>	nitric oxide synthase 2, inducible	NOS-II, Nos-2, Nos2a, i-NOS, iNOS	Mm00440502_m1	Oxidative stress. Macrophage	66
<i>Pck1</i>	phosphoenolpyruvate carboxykinase 1, cytosolic	AI265463, PEPCK, Pck-1	Mm01247058_m1	Cancer kinase	61
<i>Pd1</i>	programmed cell death 1	Pdcd1, Ly101, PD-1, Pdc1	Mm01285676_m1	Apoptosis	64
<i>Pgf</i>	placental growth factor	AI854365, PIGF, Plgf	Mm00435613_m1	Growth factor	75
<i>Smad4</i>	SMAD family member 4	AW743858, D18Wsu70e, DPC4, Madh4	Mm03023996_m1	TGF-beta signaling	71
<i>Stat3</i>	signal transducer and activator of transcription 3	1110034C02Rik, AW109958, Aprf	Mm01219775_m1	Signaling	75
<i>Vegf</i>	vascular endothelial growth factor A	Vegf, Vpf, Vegfa	Mm00437306_m1	Growth factor	61

Supporting information 4 (continued): List of gene names and probes.



Supporting information 5: Spleen weights (**A**) and liver weight (**B**). $n=5-6$. Mean and SEM. Statistical significance used Kruskal-Wallis test: $*p<0.01$, $**p<0.005$, $***p<0.001$, $****p<0.0005$.

Supporting information 6: Observed lesions in H&E stained liver [at 24 weeks of age](#)

Treatment	HCC	High grade dysplasia	Low grade dysplasia	Small cell change	Focal fatty change
Control	0	0	0	0	0
Control	0	0	0	0	0
Control	0	0	0	0	0
Control	0	0	0	0	0
Control	0	0	0	0	0
DEN/TAA	0	0	0	1	0
DEN/TAA	0	0	2	1	0
DEN/TAA	0	5	3	2	0
DEN/TAA	0	1	2	1	0
DEN/TAA	1	1	0	3	0
DEN/TAA/HFD	3	2	1	0	0
DEN/TAA/HFD	1	1	1	0	3
DEN/TAA/HFD	1	0	2	0	0
DEN/TAA/HFD	0	3	2	0	0
DEN/TAA/HFD	3	5	1	0	0
DEN/TAA/HFD	1	1	4	0	0

HCC, dysplasia, small cell change, and focal fatty change are illustrated in **Figure 3**.